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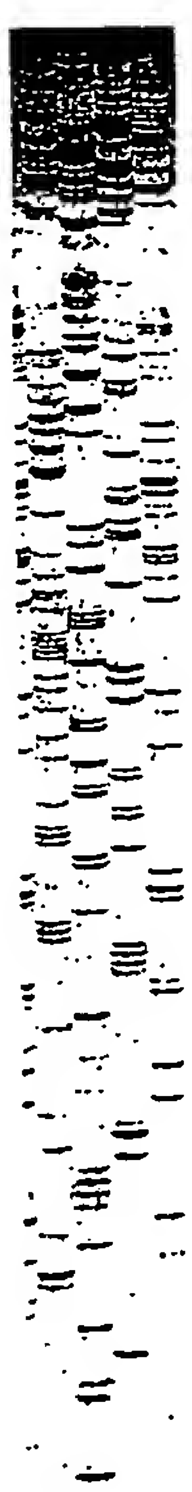
(54) Title: DNA SEQUENCING METHOD WHICH EMPLOYS VARIOUS DNA POLYMERASES AND KIT USED FOR THE SAME



(57) Abstract: The present invention relates to a DNA nucleotide sequence analysis method by means of using dideoxy nucleotide-mediated chain termination reaction and to a kit used for the same, and more particularly, directed to a DNA nucleotide sequence analysis method of by using various DNA polymerases of which affinities to dideoxynucleotide are different from each other.

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DNA SEQUENCING METHOD WHICH EMPLOYS VARIOUS DNA POLYMERASES AND KIT USED FOR THE SAME

Technical Field

The present invention relates to DNA nucleotide sequence analysis methods and kits used for the same, more particularly, to DNA nucleotide sequence analysis methods and kits for analyzing full length nucleotide sequence of DNA more accurately than the conventional methods through one time analysis of DNA nucleotide sequence analysis.

Background Art

As is known, Sanger dideoxynucleotide-mediated chain termination method is the conventional method for analyzing DNA nucleotide sequence. In Sanger method, DNA nucleotide chains propagate through the reaction with deoxynucleotide (dNTP) which contains hydroxyl group substituted at C-3 position of pentose and are terminated through the reaction with dideoxynucleotide (ddNTP) which does not contains hydroxyl group substituted at C-3 position of pentose.

In Sanger method, 4 kinds of dNTP, such as deoxyguanosinetriphosphate (dGTP), deoxyadenosinetri phosphate (dATP), deoxytymidinetriphosphate (dTTP) and deoxycytidinetriphosphate (dCTP), are used as substrates which generate DNA fragments complementary to template DNA, and 4 kinds of ddNTP, such as dideoxyguanosinetriphosphate (ddGTP), dideoxyadenosine triphosphate (ddATP), dideoxytymidinetriphosphat (ddTTP) and dideoxycytidinetri phosphate (ddCTP), are used as substrates which terminate chain propagation reaction of

complementary DNA fragments. DdNTP does not contain hydroxy group at the C-3 position of pentose, differently from dNTP. Therefore, in case that ddNTP is reacted with the end of complementary DNA fragments which are under propagation, the chain propagation reactions of complementary DNA fragments are terminated.

Therefore, in Sanger method, DNA fragments in various lengths of which the end are terminated with ddNTP, are generated. In the Sanger method, various kinds of complementary DNA fragments which correspond to the number of nucleotides of template DNA, are generated, and then are separated in order of molecular weight by electrophoresis. Thereafter, the nucleotide sequences of template DNA are recognized by determination of the terminal base of each complementary DNA fragments.

However, despite of the convenience of Sanger method, it has been a drawback that DNA in length of only 500 to 700bps can be determined accurately due to the limitation of processivity of complementary DNA propagation reaction. For example, in order to recognize accurately and completely human cDNA of which average length is 2Kb, DNA sequencing procedure should be repeated more than three times by means of partition of human cDNA. As explained above Sanger method is time-consuming, very laborious and expensive process to be employed as a sequencing method for DNA in large length.

Meanwhile, in the so-called Shot gun method which has been known as a large scale nucleotide sequencing method for genomic DNA, full length DNA is partitioned into several DNA fragments and the sequence of base of each fragments are recognized separately. Thereafter, the sequence of each fragments are compared to each other by using computer, and thereby, full length DNA sequence can

be analyzed by deletion of overlapping part. In the above Shot gun method, the time and labor required for analysis of full length DNA sequence can be reduced by means of the expansion of DNA length which can be recognized through one time analysis of DNA sequence.

In general, in the conventional Sanger dideoxy nucleotide-mediated chain termination method, single DNA polymerase is employed. Thus, the short DNA fragments which correspond to 20 to 30bps of template DNA and the long DNA fragments which correspond to 600 to 700bps of template DNA, are generated in small amounts, whereas DNA fragments which correspond to 40 to 500bps of template DNA are generated in large amounts. Therefore, the concentration of short DNA fragments and long DNA fragments are relatively low and consequently, the nucleotide sequence of terminal portions of both ends of DNA are difficult to be determined than that of middle portion of DNA.

On the reasons of the above, the length of DNA which can be analyzed through one time analysis of nucleotide sequence is limited substantially. Therefore, various researches for new method which can expand the length of DNA that can be recognized completely through one time analysis of nucleotide sequence by means of more accurate determination of the terminal portions of both ends of template DNA, have been tried for a long time.

Disclosure of Invention

Therefore, the object of the present invention is to provide a method which can determine more longer sequence of DNA through one time analysis of nucleotide sequence and a kit to be used for the method. The method of the present invention is an improvement of the conventional

DNA sequencing method of Sanger, which can be applied for determining more longer DNA than that can be determined by Sanger method.

The object of the present invention can be achieved by providing a DNA sequence analysis method which employs more than two kinds of DNA polymerases which comprise DNA polymerase of which affinity to ddNTP is higher than that of DNA polymerase which has been used in the conventional Sanger method, and DNA polymerase of which affinity to ddNTP is lower than that of DNA polymerase which has been used in the conventional Sanger method.

More specifically, the DNA polymerase of which affinity to ddNTP is higher than that of DNA polymerase used in Sanger method, generates relatively short length DNA fragments in large amount, whereas the DNA polymerase of which affinity to ddNTP is lower than that of DNA polymerase used in Sanger method, generates relatively long length DNA fragments in large amount. Therefore, by the method of the present invention which employes concurrently various DNA polymerases of which affinity to ddNTP are different from each other, DNA fragments in various length which come up to 10bps to more than 1,000bps, can be obtained indiscriminately. Consequently, DNA sequence analysis on more longer length of DNA fragments than that can be analyzed by the conventional Sanger method, is possible by the method of the present invention.

The expression, "affinity of a DNA polymerase to a dNTP or a ddNTP" used in the present invention means that the relative value which represents the extent of contribution of a specific DNA polymerase for the frequency of reaction of ddNTP or dNTP to an end of DNA fragments which are under propagation. In the present

invention, the affinity to ddNTP or dNTP of Top™ DNA polymerase manufactured by BIONEER CORPORATION in Korea, is applied as criteria of affinity to ddNTP or dNTP.

Brief Description of the Drawings

The above objects and other advantages of the present invention will become more apparent by describing in detail a preferred embodiment thereof with reference to the attached drawings, in which:

FIG. 1 is the photograph of electrophoresis of DNA fragments generated by using the conventional DNA polymerase of which affinity to dNTP is 3,000 times of affinity to ddNTP.

FIG. 2 is the photograph of electrophoresis of DNA fragments generated by using the DNA polymerase, of which affinity to dNTP is 0.5 times of affinity to ddNTP.

FIG. 3 is the photograph of electrophoresis of DNA fragments generated by using the DNA polymerase, of which affinity to dNTP is 8,000 times of affinity to ddNTP.

FIG. 4 is the photograph of electrophoresis of generated by using the DNA polymerases mixture which comprise concurrently three kinds of DNA polymerases of FIG. 1 to FIG. 3, of which affinities to ddNTP are different from each other.

FIG. 5 is the photograph of electrophoresis of generated by using the DNA polymerase mixture which comprise concurrently two kinds of various DNA polymerases of FIG. 2 and FIG. 3, of which affinities to ddNTP are different from each other.

Best Mode for Carrying Out the Invention

Hereinafter, the present invention will be described in more detail.

In the conventional Sanger method, DNA polymerase of which affinity to dNTP is about 3,000 times of affinity to ddNTP, has been used.

The method of the present invention, an improvement of Sanger method, is characterized in that the DNA polymerase mixture which comprises concurrently the DNA polymerase of which ratio of the affinity to ddNTP to the affinity to dNTP is higher than that of DNA polymerase conventionally used in Sanger method and the DNA polymerase of which ratio of the affinity to ddNTP to the affinity to dNTP is lower than that of DNA polymerase conventionally used in Sanger method, are used.

The ratio of the affinity to dNTP to the affinity to ddNTP of DNA polymerase that used in DNA sequencing analysis of the conventional Sanger method, may stand for the ratio between the reaction frequency for chain propagation by incorporation of dNTP to DNA fragment terminal catalyzed by the DNA polymerase and the reaction frequency for chain termination by incorporation of ddNTP to DNA fragment terminal catalyzed by the DNA polymerase.

In the method of the present invention, the DNA polymerase which contribute the dNTP incorporation reaction frequency which is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of the ddNTP incorporation reaction frequency which contribute to chain termination of DNA fragments under propagation, and DNA polymerase which contributes the dNTP incorporation reaction frequency which is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of the ddNTP incorporation reaction frequency which contribute to chain termination of DNA fragments under propagation, are used concurrently.

Therefore, the method of the present invention comprises:

i) a step for the preparation of nucleotide mixture which comprises concurrently the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of affinity to ddNTP and DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of affinity to ddNTP;

ii) a step for generating complementary DNA fragments by addition of template DNA along with primer into said nucleotide mixture; and

iii) a step for recognizing the terminal base of said complementary DNA fragments separated in order of molecular weight to determine the nucleotide sequence of template DNA.

The another object of the present invention, the DNA sequencing kit is composed of 4 kinds of airtight containers filled with nucleotide mixture along with the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of the affinity to ddNTP and DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of the affinity to ddNTP.

In more detail, the kit of the present invention comprises:

i) an airtight container which contains ddATP, dATP, dGTP, dCTP, dTTP, buffer solution, stabilizer, the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more

preferably not more than 0.5 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of the affinity to ddNTP;

ii) an airtight container which contains ddGTP, dATP, dGTP, dCTP, dTTP, buffer solution, stabilizer, the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of the affinity ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of the affinity to ddNTP;

iii) an airtight container which contains ddCTP, dATP, dGTP, dCTP, dTTP, buffer solution, stabilizer, the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of the affinity ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, more preferably not less than 8,000 times of the affinity to ddNTP; and

iv) an airtight container which contains ddTTP, dATP, dGTP, dCTP, dTTP, buffer solution, stabilizer, the DNA polymerase of which affinity to dNTP is less than 3,000 times, preferably not more than 1,000 times, more preferably not more than 0.5 times of the affinity ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times, preferably not less than 5,000 times, not less preferably more than 8,000 times of the affinity to ddNTP.

Hereinafter, the present invention will be described

in greater detail with reference to the following examples. The examples are given for illustration of the invention and not intended to be limiting the present invention.

Example 1.

The mixture of Thermo Sequenase(manufactured by USB company) DNA polymerase of which affinity to dNTP is 0.5 times of the affinity of to ddNTP, and Tfi mutant DNA polymerase(described in Korean Patent Application No. 98-13408) of which affinity to dNTP is 8,000 times of the affinity to ddNTP) was added into the nucleotide mixture which contains 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 150nM of ddGTP; the nucleotide mixture which contains 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 1.754μM of ddATP; the nucleotide mixture which contains 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 3.02μM of ddTTP; and the nucleotide mixture which contains 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 1μM of ddCTP, respectively to prepare the mixtures for generation of complementary DNA fragments.

Meanwhile, Top™ DNA polymerase(produced by Bioneer corporation), of which affinity to dNTP is 3,000 times of the affinity to ddNTP was added into above described four(4) nucleotide mixtures, respectively to prepare the mixtures for generation of complementary DNA fragments for the purpose of comparison with the result of the present invention.

1.5μg of pUC 19 plasmid DNA as template DNA, M13 Universal Forward 17mer(5'-gtaaaacgacggccagt, 30pmoles) as primer and distilled water, were added into above-described three(3) kinds of mixtures, respectively to

prepare 40μg of each reaction mixtures for generating complementary DNA fragments. Thereafter, complementary DNA fragments generation reaction were repeated 30 cycles sequentially for 240 seconds at 94°C, for 30 seconds at 94°C, for 30 seconds at 50°C, for 60 seconds at 72°C, and then finally proceeded further for 300 seconds at 72°C to make DNA fragment mixtures. 40μL of Stop solutions(2.5% bromophenolblue, 2.5% xylene cyanol, 10mM NaOH) were added into each DNA fragment mixtures thus obtained to terminate the generation reaction of complementary DNA fragments. The DNA fragments thus obtained were separated by electrophoresis in order of the molecular weights thereof through polyacrylamide gel prepared by 8M Urea and 6% acrylamide. The terminal base of each DNA fragments were recognized by using the Silver-staining method(by using Silverstar staining kit produced by of Bioneer corporation).

Example 2.

10X reaction buffer solution(500mM Tris-HCl, 20mM $MgCl_2$), 5M of Betain stabilizer, 5 units(1 unit means the amounts of DNA polymerase which can polymerize 1μg of DNA for 1 hour at 37°C.) of the polymerase mixture comlosed of the 2.5 units of Thermo Sequenase and 2.5 units of *Tfi* mutant DNA polymerase and the nucleotide mixture composed of 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 150nM of ddGTP were filled into an airtight container;

10X reaction buffer solution(500mM Tris-HCl, 20mM $MgCl_2$), 5M of Betain stabilizer, 5 units of the DNA polymerase mixture composed of 2.5 units of Thermo Sequenase and 2.5 units of *Tfi* mutant DNA polymerase and the nucleotide mixture composed of 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 1.754μM of ddATP were

filled into an airtight container;

10X reaction buffer solution(500mM Tris-HCl, 20mM $MgCl_2$), 5M of Betain stabilizer, 5 units of the DNA polymerase mixture composed of 2.5 units of Thermo Sequenase 2.5 units of *Tfi* mutant DNA polymerase and the nucleotide mixture composed of 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 3.02μM of ddTTP were filled into in an airtight container; and

10X reaction buffer solution(500mM Tris-HCl, 20mM $MgCl_2$), 5M of Betain stabilizer, 5 units of the DNA polymerase mixture composed of 2.5 units of Thermo Sequenase 2.5 units of *Tfi* mutant DNA polymerase and the nucleotide mixture composed of 3μM of dGTP, 30μM of dATP, 30μM of dTTP, 30μM of dCTP and 1μM of ddCTP were filled into an airtight container, thus produce the DNA sequencing kit of the present invention, which comprises 4 kinds of airtight containers.

To each airtight containers of the said DNA nucleotide sequencing kit, 1.5μg of pUC 19 plasmid DNA as template DNA, M13 Universal Forward 17mer(5'-gtaaaacgacggccagt, 30pmoles) as primer and distilled water, were added to, respectively to make 40μg of the reaction mixtures for generating complementary DNA fragments. The DNA fragments thus generated, were separated, respectively in other of molecular weight thereof and then, the terminal base of the DNA fragment were analyzed according to the method of Example 1.

FIG. 1 is the photograph of electrophoresis of DNA fragments generated by using the conventional DNA polymerase (Top™ DNA polymerase, 5 units)of which affinity to ddNTP is conventional.

FIG. 2 is the photograph of electrophoresis of DNA

fragments generated by using the DNA polymerase(Tfi mutant DNA polymerase, 5 units)of which affinity to ddNTP is higher than that of conventional DNA polymerase.

FIG. 3 is the photograph of electrophoresis of DNA fragments generated by using the DNA polymerase(Thermo Sequenase, 5 units), of which affinity to ddNTP is lower than that of conventional DNA polymerase.

FIG. 4 is the photograph of electrophoresis of generated by using the DNA polymerases mixtures composed of three kinds of DNA polymerases of FIG. 1 to FIG. 3(2unit, 2unit, 1unit, respectively) of which affinities to ddNTP are different from each other.

FIG. 5 is the photograph of electrophoresis of generated by using the DNA polymerases mixtures of the present invention composed of two kinds of DNA polymerases of FIG. 2 and FIG. 3(2.5unit, 2.5unit, respectively), which have different affinities to ddNTP.

Industrial Applicability

As described in above, the nucleotide sequence of DNA of 10 to 1,000 bps can be analyzed more accurately and completely by method or by using the Kit of the present invention than by the conventional Sanger method. Consequently, it is possible to determine DNA sequence in more longer length than that can be determined by Sanger method through one time analysis of nucleotide sequence.

What is claimed is

1. A DNA nucleotide sequence analysis method which employes the dideoxynucleotide-mediated chain termination reaction, characterized in comprising:
i) a step for preparing nucleotide mixture that comprises concurrently the DNA polymerase of which affinity to dNTP is less than 3,000 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times of the affinity to ddNTP;
ii) a step for generating DNA fragments by adding a template DNA along with a primer into said nucleotide mixture; and,
iii) a step for determining the nucleotide sequence of the template DNA by recognizing terminal bases of said DNA fragments separated in order of molecular weight thereof.

2. The DNA nucleotide sequence analysis method according to claim 1, wherein the nucleotide mixture comprises concurrently the DNA polymerase of which affinity to dNTP is not more than 1,000 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is not less than 5,000 times of the affinity to ddNTP.

3. The DNA nucleotide sequence analysis method according to claim 1, wherein the nucleotide mixture comprises concurrently DNA polymerase of which affinity to dNTP is not more than 0.5 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is not less than 8,000 times of the affinity to ddNTP.

4. The DNA nucleotide sequence analysis method

according to claim 1, wherein the nucleotide mixture further comprises the DNA polymerase of which affinity to dNTP is 3,000 times of the affinity to ddNTP.

5. The DNA nucleotide sequence analysis method according to claim 2, wherein the nucleotide mixture further comprises the DNA polymerase of which affinity to dNTP is 1,000 to 5,000 times of the affinity to ddNTP.

6. The DNA nucleotide sequence analysis method according to claim 3, wherein the nucleotide mixture further comprises the DNA polymerase of which affinity to dNTP is 0.5 to 8,000 times of the affinity to ddNTP.

7. The DNA nucleotide sequence analysis method according to claim 1, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

8. The DNA nucleotide sequence analysis method according to claim 1, wherein the terminal base of each DNA fragments are recognized by silver staining method.

9. The DNA nucleotide sequence analysis method according to claim 2, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

10. The DNA nucleotide sequence analysis method according to claim 2, wherein the terminal base of each DNA fragments are recognized by silver staining method.

11. The DNA nucleotide sequence analysis method

according to claim 3, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

12. The DNA nucleotide sequence analysis method according to claim 3, wherein the terminal base of each DNA fragments are recognized by silver staining method.

13. The DNA nucleotide sequence analysis method according to claim 4, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

14. The DNA nucleotide sequence analysis method according to claim 4, wherein the terminal base of each DNA fragments are recognized by silver staining method.

15. The DNA nucleotide sequence analysis method according to claim 5, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

16. The DNA nucleotide sequence analysis method according to claim 5, wherein the terminal base of each DNA fragments are recognized by silver staining method.

17. The DNA nucleotide sequence analysis method according to claim 6, wherein the DNA fragments are separated in order of molecular weight thereof by electrophoresis.

18. The DNA nucleotide sequence analysis method according to claim 6, wherein the terminal base of each

DNA fragments are recognized by silver staining method.

19. A DNA nucleotide sequencing kit composed of an airtight container filled with reaction buffer, stabilizer, dATP, dGTP, dCTP, dTTP, ddATP and DNA polymerase; an airtight container filled with reaction buffer, stabilizer, dATP, dGTP, dCTP, dTTP, ddGTP and DNA polymerase; an airtight container filled with reaction buffer, stabilizer, dATP, dGTP, dCTP, dTTP, ddCTP and DNA polymerase; and an airtight container filled with reaction buffer, stabilizer, dATP, dGTP, dCTP, dTTP, ddTTP and DNA polymerase, which is characterized in that said DNA polymerase is the mixture of the DNA polymerase of which affinity to dNTP is less than 3,000 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is higher than 3,000 times of the affinity to ddNTP.

20. The DNA nucleotide sequencing kit according to claim 19, wherein said DNA polymerase mixture is composed of the DNA polymerase of which affinity to dNTP is not more than 1,000 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is not less than 5,000 times of the affinity to ddNTP.

21. The DNA nucleotide sequencing kit according to claim 19, wherein said DNA polymerase mixtures is composed of the DNA polymerase of which affinity to dNTP is not more than 0.5 times of the affinity to ddNTP and the DNA polymerase of which affinity to dNTP is not less than 8,000 times of the affinity to ddNTP.

22. The DNA nucleotide sequencing kit according to claim 19, wherein said DNA polymerase mixture further

comprises the DNA polymerase of which affinity to dNTP is 3,000 times of the affinity to ddNTP.

23. The DNA nucleotide sequencing kit according to claim 20, wherein said DNA polymerase mixture further comprises the DNA polymerase of which affinity to dNTP is 1,000 to 5,000 times of the affinity to ddNTP..

24. The DNA nucleotide sequencing kit according to claim 21, wherein said DNA polymerase mixture further comprises the DNA polymerase of which affinity to dNTP is 0.5 to 8,000 times of the affinity to ddNTP.

FIG. 1

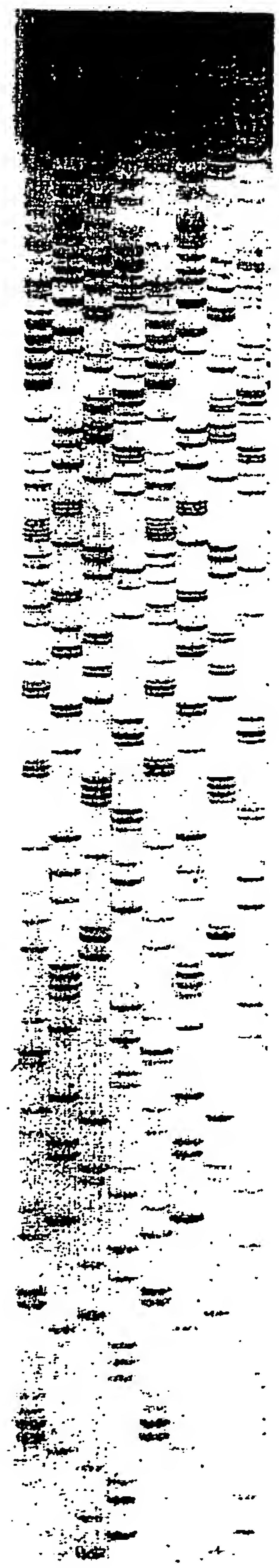


FIG. 2

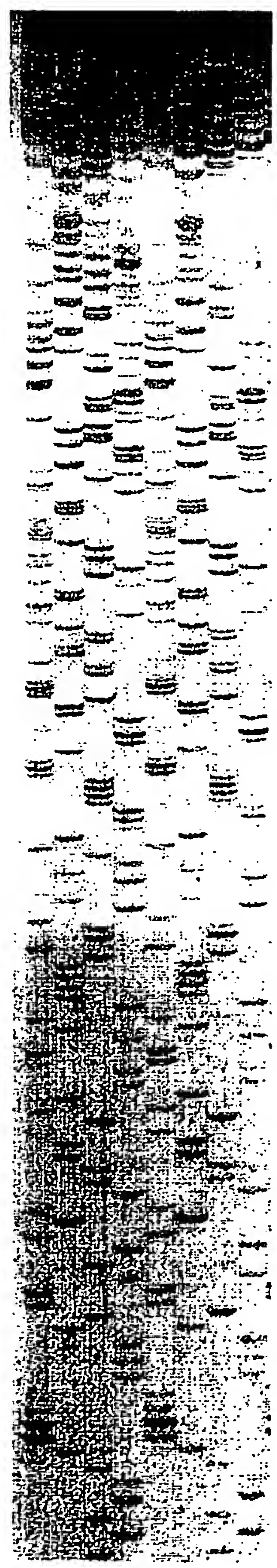


FIG. 3

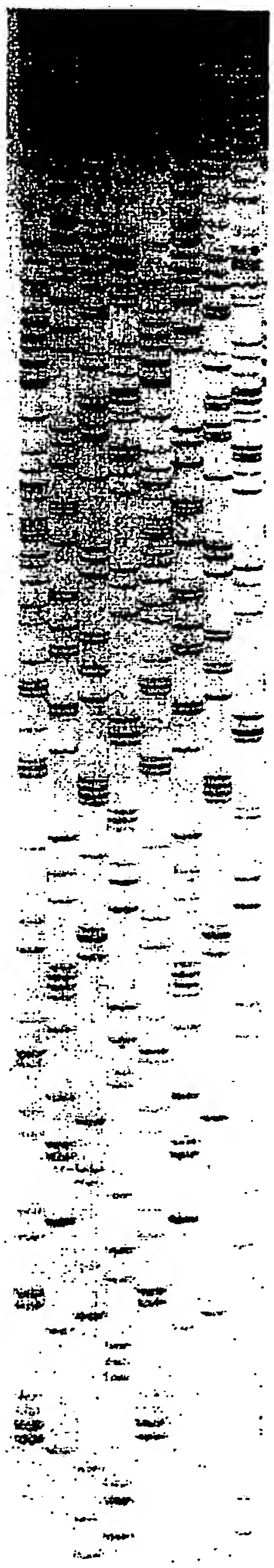


FIG. 4

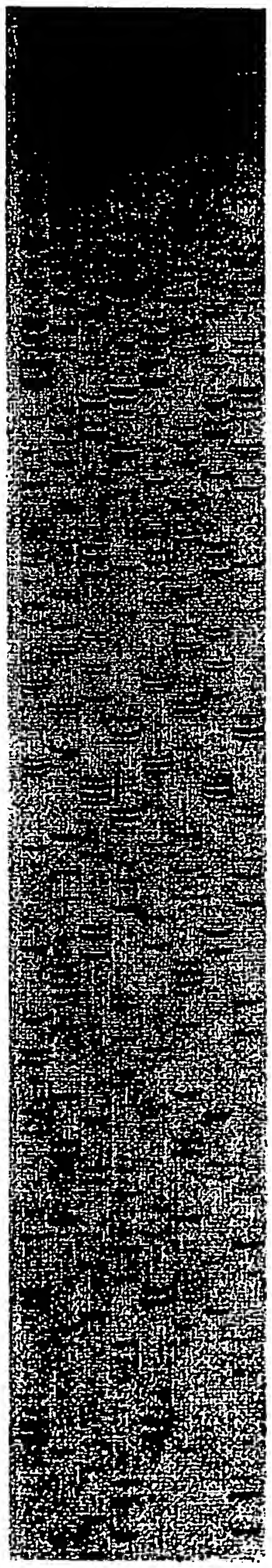
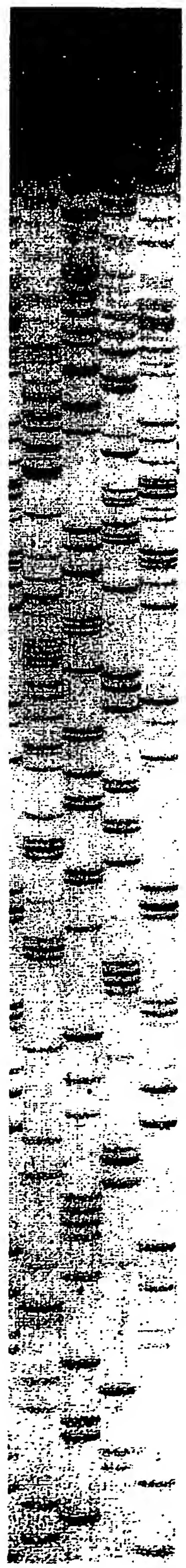


FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 00/01354

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/42348 A1 (SEQUENOM, INC.) 13 November 1997 (13.11.97) claims 1,3,17,18.	1,2,4,5,7-10,13-16, 19,20,22,23
X	EP 0854196 A1 (BOEHRINGER MANNHEIM GMBH) 22 July 1998 (22.07.98) page 4, lines 12-25; claims 1-4, 22, 31, 32.	1,2,4,5,7-10, 13-16,19,20, 22,23

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2001 (26.03.2001)	11 April 2001 (11.04.2001)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer MOSSER Telephone No. 1/53424/437

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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